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(57) Abstract

The present invention refers to the use of temporal thermal gradients for the separation of DNA fragments, amplified by PCR, both normal and containing point mutations, by capillary zone electrophoresis in presence of viscous polymer solutions. In this system the temperature control in the capillary is obtained via dedicated software and the fragments are revealed either by their natural UV absorbance at 254 nm or by laser induced fluorescence. It is also possible to operate with viscous solutions of polyacrylamides, in particular polyacrylamides containing N-substituted monomers, such as N-methylacrylamide and N-acryloyl amino ethoxy ethanol. Methods are described for producing short-chain polyacrylamides of low viscosity, which can be replaced after each run.

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SEPARATION OF NUCLEIC ACIDS BY CAPILLARY ELECTROPHORESIS IN THERMAL GRADIENTS IN VISCOUS POLYMER SOLUTIONS

The present invention refers to the use of thermal gradients (coupled, when needed, to chemical denaturants) in time (as opposed to thermal gradients in space), for the separation of PCR-amplified DNA fragments, both normal or containing point mutations, via capillary zone electrophoresis in presence of viscous polymer solutions (either linear or branched). The present invention comprises also means for controlling the temperature from within, *via* the use of dedicated software calculating the real temperature inside the capillary with a precision >1°C. The present invention comprises also the use of batteries of capillaries, with the possibility, when needed, of an individual control of voltage on each capillary, so as to be able to operate under different thermal gradients according to the type of DNA under separation. The present invention furthermore comprises DNA detection via laser induced fluorescence. It also includes the possibility of operating with a variety of polymer solutions, as typically used in DNA fractionations (including, but not limited to, polyacrylamides, agarose, hydroxyethyl cellulose, hydroxypropyl methyl cellulose, methyl cellulose, dextran, pullulan, polyethylene glycol, polyethylene oxide, polyvinyl pyrrolidone, glucomannan), either alone or in mixtures, and/or with polyacrylamides made of hydrolysis-resistant monomers (typically N-substituted, such as N-methyl acrylamide and N-acryloyl amino ethoxy ethanol).

Additionally, the present invention describes the synthesis of short-chain polyacrylamides (optimized for DNA fragment separation) via two different processes: a) cavitation of long-chain polyacrylamides by sonication and b) polymerization in presence of inhibitors (e.g., isopropanol) and at high temperatures. With this latter process it is possible to obtain polyacrylamides of much reduced viscosities and molecular mass values, highly performing in the 100 to 1000 bp interval, *i.e.* in the interval of greatest interest for analysis and detection of genetic mutations.

A number of patents have already been published for DNA analysis in capillary zone electrophoresis (CZE). E.g., C. Fujimoto (Jap. Pat. No. 92208382 & 9383747) has described a system of two co-axial capillaries for separation of DNA and proteins. Demorest, Werner, Wiktorowicz, Oaks & Wenz (US Pat. Nos. 5015350; 5181999; 5264101) have described an electrophoresis system in which the capillary is filled with a buffer containing 0.05 to 30% of an un-cross-linked, neutral and hydrophilic polymer, having Mr values from 20 to 50.000 kDa, admixed to small amounts of a charged, hydrophilic polymer (from 0.01 to 1%). This system allows the separation of biopolymers, e.g. proteins, nucleic acids and oligosaccharides by CZE. The preferred (co)polymers are: polyacrylamide, polyoxides, polyethers, vinyl polymers, acrylic polymers, cellulosic polymers, polysaccharides and vegetable gums. Huang, Mathies and Quesada (US Pat. No. 5274240) have patented a battery of capillaries, mounted on a moving platform, able to perform the simultaneous analysis of a minimum of 20

samples containing DNA fragments, bearing a fluorescent tag, via excitation with laser beams of appropriate wavelength. The separation occurs in polyacrylamide gels or in viscous solutions of polyacrylamides and the main application is for DNA sequencing. Lux, McManigill & Young (US Pat. No. 5180475) have proposed a novel method for controlling the electrosmotic flux (EEO) in capillaries, consisting in generating a second voltage gradient, perpendicular to the axial voltage gradient utilized for analyte separation, in the radial direction. With this radial gradient it is possible to control (and even eliminate) the EEO flux and thus to ameliorate separation of proteins, DNA and RNAs. In another application, Jamkbara (US Pat. No. 5277780) proposed a battery of gel-filled capillaries for DNA separation and fluorescent detection. Chin (US Pat. 5110424) proposed a method for DNA fractionation consisting in filling the capillary with a 5000 Da polymer moving, by EEO flux, in an opposite direction to the DNA migration. Selectivity could thus be modulated by reducing the difference between the velocity of the EEO flux and the DNA fragment velocity.

Two patent applications, in particular, refer to the possibility of operating with thermal gradients in CZE. In one, by the title: "Buffer gradient and temperature gradient capillary electrophoresis" (Weinberger & Gassmann, US Pat. 5047134) the possibility of creating a buffer gradient, via two buffer reservoirs connected by a mixing chamber to the separation capillary, is proposed. A method is additionally proposed for controlling the capillary temperature, In a

second patent, by the title: "Thermal control for capillary electrophoresis apparatus", Weinberger & Mills (US Pat. No. 5066382) propose temperature control via a thermistor placed outside and in contact with the capillary. The final means for obtaining a given temperature is by recirculating air at the outside, at the desired temperature, so as to subtract or add temperature to the capillary. Such means for temperature control include additionally Peltier elements. It is moreover proposed to determine the inner capillary temperature via measurements of the electric resistance of the capillary chamber at predetermined voltage values.

In the analysis of inherited genetic diseases and for the mass screening of genetic mutations, it is of fundamental importance to be able to resolve, within an electrophoretic run, single or multiple DNA point mutations. Up to the present, the most common method has been the one described by Fischer & Lerman (*Proc. Natl. Acad. Sci. USA* 80, 1983, 1579-1583), consisting in denaturing gradient gel electrophoresis (DGGE). DGGE is based on the principle that the mobility of a partially melted DNA double strand is markedly reduced compared to that of an intact ds-DNA. The sequences amenable to separation consist of two domains, having a low and a high melting temperature (T_m). This allows, within a narrow range of concentrations of denaturant (be it a chemical or temperature), to obtain fusion intermediates containing single and double-stranded DNAs. When mixtures of such molecules, all having the same length but differing by a point mutation, migrate in a gel in

presence of a denaturing gradient, separation will occur along the electrophoretic path due to different equilibria among native, melted and partially melted species. In general, the partially melted species will migrate more slowly than the fully native (double stranded) form, since the radius of gyration of the former is larger than that of the latter, so that the frictional resistance to migration will be greater in the partially melted form. In a variant of this method, the mutated DNA chains are mixed with normal (wild-type) DNA chains and hetero-duplexes are formed by melting the mixture above the T_m of the highest melting domain and subsequent reannealing by cooling. These hetero-duplexes have T_m values in general lower than the T_m of homo-duplexes, due to uncoupling of bases in the region of the mutation. When mixtures of hetero- and homo-duplexes are forced to migrate in a temperature gradient, separation will ensue due to the different T_m values. In fact, a classic variant of the Fischer & Lerman method, in which only gradients of chemical denaturants are used (typically urea and formamide), is electrophoresis in thermal gradients. In this method (Riesner, Henco & Steger, *Advan. Electr.* 4, 1991, 171-250) electrophoresis is conducted in a gel slab to which extremities a temperature gradient (e.g. from 30 to 90°C) is applied, perpendicular or parallel to the migration direction.

In the present invention, we demonstrate the possibility, in CZE, of creating temporal thermal gradients (as opposed to the gradients of Riesner *et al.*, which exist only in the space) for separating such point mutations in DNA fragments. Also comprised in the

present invention are:

(a) the use of viscous polymer solutions, which exert sieving in nucleic acids on the basis of different radii of gyration of native and partially melted DNA molecules;

(b) the possibility of using batteries of capillaries, for multiple analyses;

(c) the possibility of revealing DNA fragments via laser induced fluorescence;

(d) the possibility of including in the CZE unit a thermal cycler, for *in situ* amplification of the DNA fragments to be then analyzed by CZE; such amplification being conducted, as needed, either in a microtiter plate or directly in a specific region inside the capillary;

(e) the possibility of performing such analysis not only in fused silica capillaries, as routinely used in CZE technology, but also in chips, containing microetched channels.

Included into the present invention is also the possibility of an individual control of the voltage gradient for modulating the temperature on each capillary, and the use of dedicated computer programs for determining the temperature inside a capillary. Included in the present invention is also the combined use of denaturing gradients, such as the simultaneous use of chemical denaturants (such us, but not exclusively, urea and formamide) with temperature denaturation. This combined use allows in fact reaching temperatures, inside the capillary, well below the boiling temperature of the solvent (in general, but not limited to, water).

The present invention differs from the temperature control of capillaries, as reported in the above patents by Weinberger & Gassmann and by Weinberger & Mills, in several fundamental points:

(a) first of all, the temperature control method, reported in the above patents, is an "external method", consisting in measuring the temperature outside the capillary, and then in modulating its temperature by recycling cold or warm air. However, it has been demonstrated (M.S. Bello, P. de Besi & P.G. Righetti, *J. Chromatogr.* 652, 1993, 329-336) that the steady-state temperature inside a capillary can be substantially different from the outside temperature, due to inertia in dissipating heat from the thick silica wall and the polyimide coating. This difference could be as high as 40-50°C, thus incompatible with a reproducible separation of point mutations of nucleic acids, where the temperature control during the electrophoretic run should be better than ±1°C. This temperature control, in the present invention, comes from "within" the capillary, via dedicated computer programs which, by assuming a linear dependence of the current on the temperature of the viscous buffer solution, and known the buffer specific conductivity, its thermal coefficient (α), the applied voltage gradient, the coefficient of heat dissipation (Biot number) and the precise capillary diameter and length, can predict the precise temperature inside the capillary to better than ±1°C. Thus, in order to vary the desired temperature inside the capillary, according to the melting temperature of the mutants under exam, it is sufficient

to vary the background buffer conductivity and/or the applied voltage (and also, if necessary, the capillary diameter).

(b) for the optimum separation of such mutants (in general, amplified DNA fragments of typical lengths of the order of 100 to 500 base pairs, bp) liquid polymers offering an optimal separation in this length window are needed. In the above patents, linear polymers of extreme length are typically employed (e.g., polyacrylamides, methyl celluloses, with typical size of the order of a few million Da). In the present invention, we demonstrate that optimal resolution is only obtained with polymer sizes of much reduced length, typically polyacrylamides of 100000 to 200000 Da in M_r or even by mixing different types of polymers (e.g., short-chain polyacrylamides and polyethylene glycols from 35000 to 100000 Da). Additionally, different methods are here reported for the synthesis of such "short-chain" polyacrylamides. One of them consists on masticating, via ultrasounds, in presence of radical scavengers, long-chain polyacrylamides to the desired chain length. In another method, polyacrylamides are polymerized in presence of "chain transfer" agents (e.g., isopropanol) and at high temperatures (e.g., 70°C), so as to produce "short-chain" polymers, endowed with low viscosity. These polyacrylamides have the advantage of combining excellent sieving properties with ease of extrusion from the capillary, due to the very low viscosity. Comprised in the present invention is also the use of polyacrylamide matrices made of hydrolytically-stable monomers, such as the novel monomer N-acryloyl amino

ethoxy ethanol (M. Chiari, C. Micheletti, M. Nesi, M. Fazio & P.G. Righetti, *Electrophoresis* 15, 1994, 177-186).

The advantages of the denaturing gradients and of the novel sieving liquid polymers cited above, as compared with presently-available systems, are illustrated below.

Separation of DNA point mutations in mixed physico-chemical denaturants

Fig. 1 shows the separation of an amplified DNA fragment (cystic fibrosis, CF, gene from a normal individual) in the absence (lower tracing) and presence (upper profile) of thermal denaturing gradients. In the lower tracing, separation occurs at constant temperature (45°C) and in presence of chemical denaturants (6 M urea). The peaks eluted between 27 and 35 min represent oligonucleotide primers. The normal amplified DNA is eluted as a single peak (labelled W_t/W_t) between 58 and 60 min. In the upper tracing, the same separation is carried out still in presence of 6 M urea (a partial denaturant of DNA) but additionally in presence of a temperature gradient with a slope of 0.15°C/min, so as to reach a maximum of 49.5°C after 30 min of electrophoresis. The shape of the thermal gradient can be visualized from the base-line ramp, due most likely to a variation of refractive index induced by the temperature ramp. It can be appreciated that the amplified DNA fragment (W_t/W_t) is eluted much earlier (in only 24 min) and remains as a single peak, since there are no mutations present in the oligonucleotide chain.

Fig. 2 shows the separation of a "hetero-polymer", comprising a normal chain and a chain containing two polymorphisms in exon 14a (V868V and T854T), amplified from a patient suffering from cystic fibrosis. In the lower tracing, the separation is again performed at constant temperature (T=45°C) and in presence of 6 M urea. The peaks eluted between 25 and 35 min represent oligonucleotide primers. The peak emerging at 38 min also represents a longer chain primer (55 bp), whereas the peak with a transit time centred at 47 min represent the hetero-polymer, comprising a normal and a mutant chain (W_t/M). When the same separation is conducted again in 6 M urea, but in presence of a temperature gradient having a slope of 0.15°C/min, so as to reach a maximum of 49.5°C after 30 min of electrophoresis, partial melting of the different homo- and hetero-polymers present in the sample occurs. As a result of that, in the upper tracing of Fig. 2, one can note that the single peak obtained in the constant temperature run is now resolved into four peaks, representing: 1: the mutated homo-polymer (M/M); 2: the normal homo-polymer (W_t/W_t); 3: the hetero-polymer of the type normal/mutant (W_t/M) and 4: the hetero-polymer of the type mutant/normal (W_t/M). The sum of the areas of the four peaks corresponds to the area of the single peak in the lower tracing.

The present technique can not only resolve low melters (as in Figs. 1 & 2), starting at a temperature plateau of 45°C, but also intermediate and high melters.

Fig. 3 shows the analysis of a set of intermediate melting fragments, amplified from CF patients

heterozygous for different mutations in exon 11 of the CFTR gene: 1717-1G --> A (panel A); G542X (G --> T at 1756; panel C) and 1784delG (panel D) with their respective normal control (panel E). All mutants exhibit the characteristic four-peak profile, *vs.* a single band in the control. As shown in the temperature profile of panel B, these mutants are intermediate melters, with T_m's in the 56.5 to 57.8°C range.

Fig. 4 shows the optimized condition set up for a higher melting fragment, amplified from a CF patient homozygous for the M1V mutation (A --> G transversion at position 133 in exon 1 of the CFTR gene). The panel shows the electropherogram of the sample injected at a constant temperature plateau (65°C), constant denaturant buffer, but in the absence of a temperature gradient. We observe separation between homo- and hetero-duplexes, but not within each other. The group of peaks eluting from 35 to 48 min corresponds to unpurified primers with an without GC-clamps. The insert shows the optimized separation in a 65 to 67°C gradient with a slope of 0.1°C/min: the correct spectrum of four bands is now obtained.

The temperature is the one truly existing inside the capillary and is precisely determined with the aid of computer programs developed by us (M.S. Bello, E.I. Levine & P.G. Righetti: Computer assisted determination of the inner temperature and peak correction for capillary zone electrophoresis. *J. Chromatogr.* 652, 1993, 329-336).

Use of masticated and "chain-transfer" polyacrylamides.

The production of masticated polyacrylamides allows

the synthesis of chains having drastically reduced viscosities due to the marked decrements of the average chain size of the polyacrylamide polymer, which decreases from >2 million Da to *ca.* 550000 Da.

Fig. 5 shows the progressive decrements of viscosity and average molecular mass of polyacrylamides during the mastication process by sonication. The viscosity has been measured with a Bohlin VOR rheometer (Bohlin Rheology, Lund, Sweden), whereas M_r has been determined by gel permeation (HPLC Waters' 590 Solvent Delivery System, equipped with two Waters Ultrahydrogel columns and with a differential refractometric detector R401 against polyethylene glycol standards.

Such reduced viscosity allows the injection into the capillary of much more concentrated solutions of polyacrylamides (up to 10%), which in turn permit optimization of resolution in the DNA size interval (typically from 100 to 500 bp) most interesting for the screening of genetic mutations via analysis of amplified DNA fragments. Even better separations can be achieved by polymerization in presence of chain-transfer agents (e.g., 3% isopropanol) coupled to elevated temperatures, a process which generates chains of further reduced lengths and viscosities.

Fig. 6 shows viscosity measurements as a function of polymer concentration obtained by polymerization in presence of "chain transfer" agents at 35°C and at 70°C. The viscosity has been measured with a Bohlin VOR rheometer (Bohlin Rheology, Lund, Swden). The drastic viscosity reduction at high temperatures is due to formation of short chains (M_r of only 180000 Da at 70°C,

as opposed to M_r of 450000 Da when polymerizing at 35°C).

As shown in Fig. 6, the viscosities of polyacrylamides polymerized at 35 or at 70°C are markedly different. In the latter case, a strong decrement of viscosity is obtained (e.g., in an 8% polymer solution, the viscosity diminishes from 450 mPa.s to barely 120 mPa.s). This strong viscosity decrement is due to a marked reduction in average chain length, which diminishes from 430000 Da (when polymerizing at 35°C) to only 180000 Da at 70°C.

Fig. 7A shows the separation of a multiplex of a series of amplified DNA fragments for the screening of different exons in the muscular distrophy gene Waters' Quanta 4000-E. Sample injection: 10 s at 6 kV. Electrophoretic buffer: 89 mM Tris-borate, 2 mM EDTA, pH 8.3. Detection at 254 nm. A; separation in linear polyacrylamides at 6%T (average M_r: >2 million Da); B: separation in 10%T linear polyacrylamides obtained by "chain transfer" synthesis at 70°C (average M_r: 180000 Da). Note the marked increment in resolution: from 11 peaks in A to 18 peaks in B (the mixture contains 18 different amplified fragments). The upper tracing in B represents the separation of 14 exons of modified deleted Chamberlains' and Beggs' mixed multiplex. The lower electropherogram in B shows the separation of 18 exons of modified non deleted Chamberlains' and Beggs' multiplex.

In a standard matrix (containing 6% polyacrylamide, in the absence of cross-linker) it isn't possible to separate more than 11 DNA peaks (although the multiplex

contains a total of 18 different fragments). It is not even possible to ameliorate the analysis by increasing the concentration of liquid polymer, since at higher concentrations the viscosity becomes so high that injection into the capillary is not any longer feasible. It was thus impossible to identify the various peaks, due to the very poor quality of the separation. On the contrary, when filling the capillary either with masticated chains, or even better with "chain-transfer" polyacrylamides (as obtained in presence of 3% isopropanol at 70°C), it was possible to separate all 18 fragments (Fig. 7B).

CLAIMS

1. A process for the separation of double-stranded nucleic acid fragments by non-micellar electrophoresis in an electrophoretic separation medium, said process comprising imparting a time-variable temperature to said separation medium to cause said double-stranded nucleic acid fragments to separate due to differing melting temperatures and the effect of melting on migration rates.

2. A process for separation of DNA fragments via CZE in temporal thermal gradients according to claim 1, characterized by the fact that the temperature inside the capillary is varied in time by applying a voltage gradient as a function of the buffer specific conductivity, of the thermal coefficient of the viscous polymer solution, of the coefficient of heat transfer, of the length and diameter of the capillary and additionally characterized by the fact of employing low-viscosity solutions of polyacrylamides (both N-substituted and un-substituted) and having M_r values in the 100000 to 200000 Da, either alone or mixed with other polymers.

3. A process according to claim 1 and 2, characterized by the fact that the polyacrylamides are constituted by monomers of the type N-acryloyl amino ethoxy ethanol or other N-substituted acrylamides.

4. A process according to claims 1, 2 e 3, characterized by the fact that the viscous sieving polymer solutions contain additionally DNA denaturing agents.

5. A process according to claim 1 and 2, characterized by the fact that the viscous sieving polymer solutions for DNA separation comprises a variety of polymer solutions, as typically used in DNA fractionations (including, but not limited to, agarose, hydroxyethyl cellulose, hydroxypropyl methyl cellulose, methyl cellulose, dextran, pullulan, polyethylene glycol, polyethylene oxide, polyvinyl pyrrolidone, glucomannan), either alone or in mixtures.

6. A process according to claims 2 or 4, characterized by the use of a battery of capillaries for the multiple screening of DNA fragments.

7. A process according to claim 6, in which the temperature in each individual capillary is controlled via dedicated software and via individual control of the applied voltage, so as to generate temporal thermal gradients of different slopes.

8. A process according to claims 2 and 5, characterized by the fact that the dedicated software for temperature control accounts for the buffer specific conductivity, its thermal coefficient, the applied voltage gradient, the coefficient of heat dissipation, the precise capillary diameter and lenght and is able to predict the precise inner temperature to better then ± 1°C

9. A process according to any of the above claims for the separation of point mutations in DNA.

10. A process according to any of the above claims in which DNA is revealed via fluorescent tags and excitation with UV/Vis and/or laser beams.

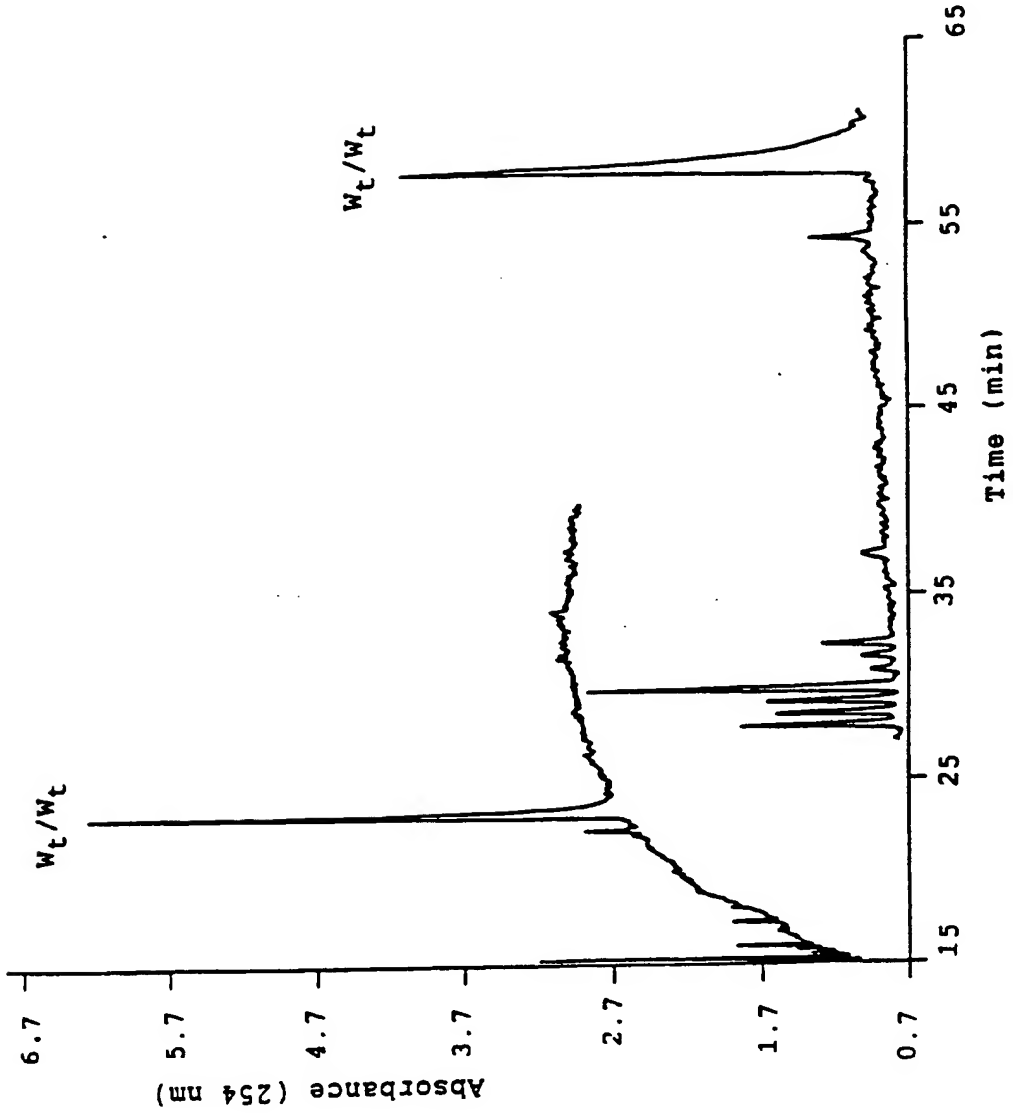
11. A process according to claims 1, 2, 6 and 10, in

which the capillary electrophoresis instrument, assembled with a battery of capillaries, able to develop thermal gradients, equipped with UV/Vis and/or laser excited fluorescence detection, is also built to contain a thermal cycler, for the *in situ* amplification of the DNA fragments to be subsequently analysed by capillary electrophoresis; such amplification occurring, if needed, directly in a specific zone inside the separation capillary.

12. A process according to claim 1 and 2, in which the separation in thermal gradients is conducted in chips (reusable or disposable), containing microetched channels.

13. A process according to claims 1 e 2, in which the separation can be conducted in discontinuous or cyclic thermal gradients in which, e.g., the heating ramp is followed by a cooling cycle and, if needed, by a new thermal ramp and additional thermal cycles, as needed for optimization of separation.

14. A process according to anyone of the above claims, comprising means and methods for achieving temporal thermal gradients by a combination of "external" temperature control, for reaching a given temperature plateau, and "internal" temperature variation as generated by ohmic heat via voltage, or conductivity, ramps.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6.7
5.7
4.7
3.7
2.7
1.7
0.7
15
25
35
45
55
65
Time (min)
W_t/W_t
W_t/W_t

Figure 1

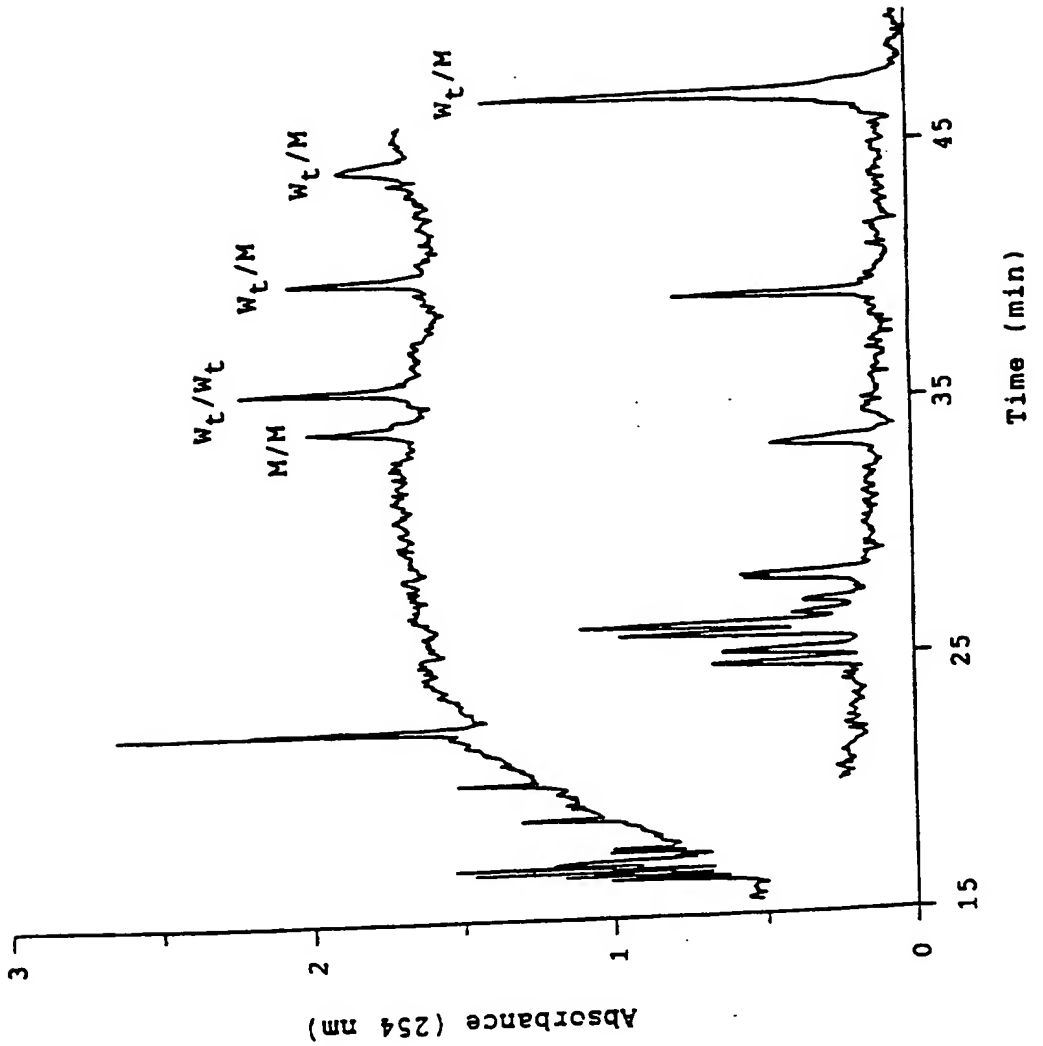

Figure 2

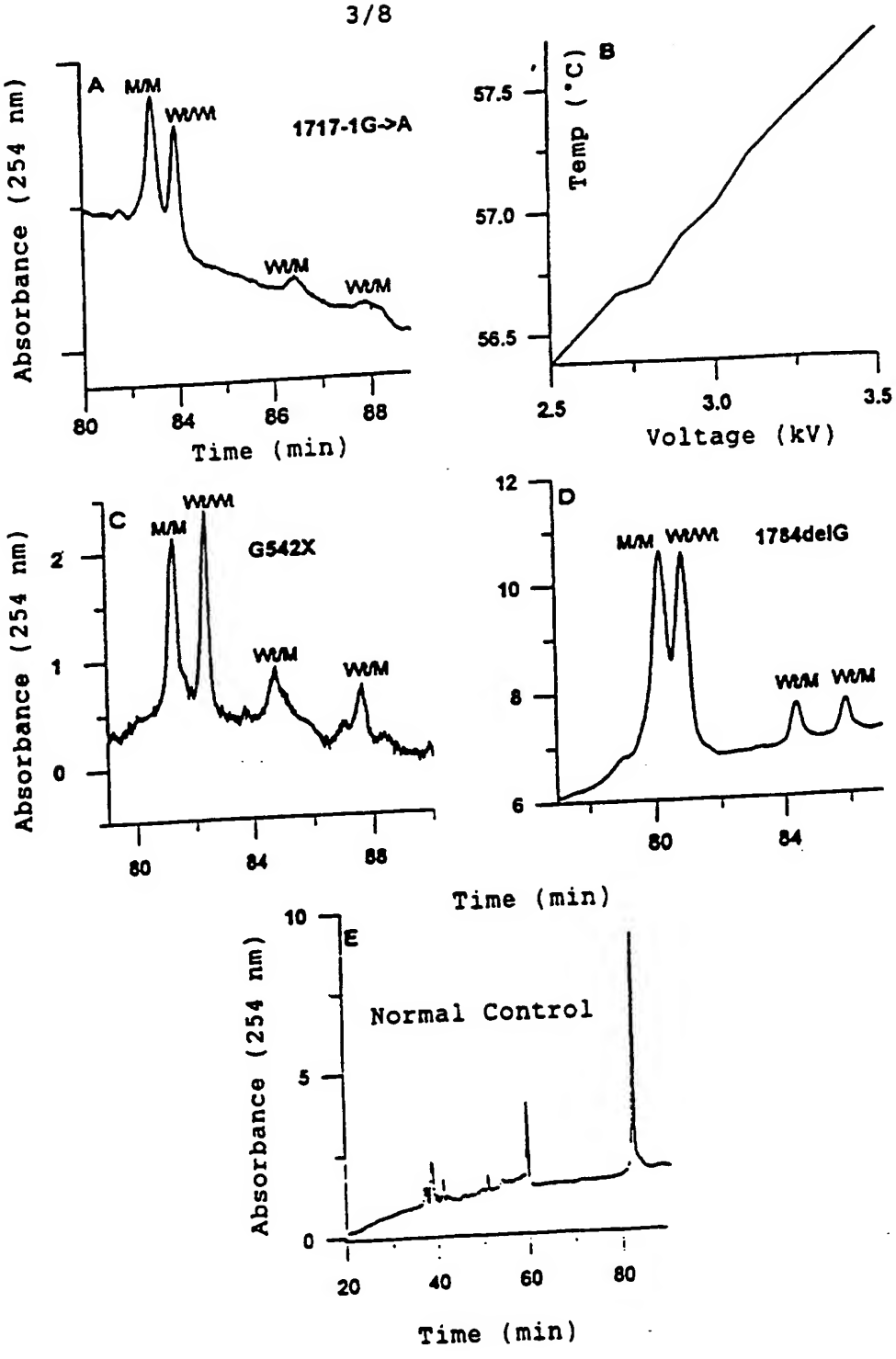
A
M/M
W/M
1717-1G->A
W/M
W/M
Absorbance (254 nm)
80
84
86
88
Time (min)
B
Temp (°C)
57.5
57.0
56.5
2.5
3.0
3.5
Voltage (kV)
C
M/M
W/M
G542X
W/M
W/M
Absorbance (254 nm)
2
1
0
80
84
88
D
M/M
W/M
1784delG
W/M
W/M
12
10
8
6
80
84
Time (min)
E
Normal Control
Absorbance (254 nm)
10
5
0
20
40
60
80
Time (min)

Figure 3

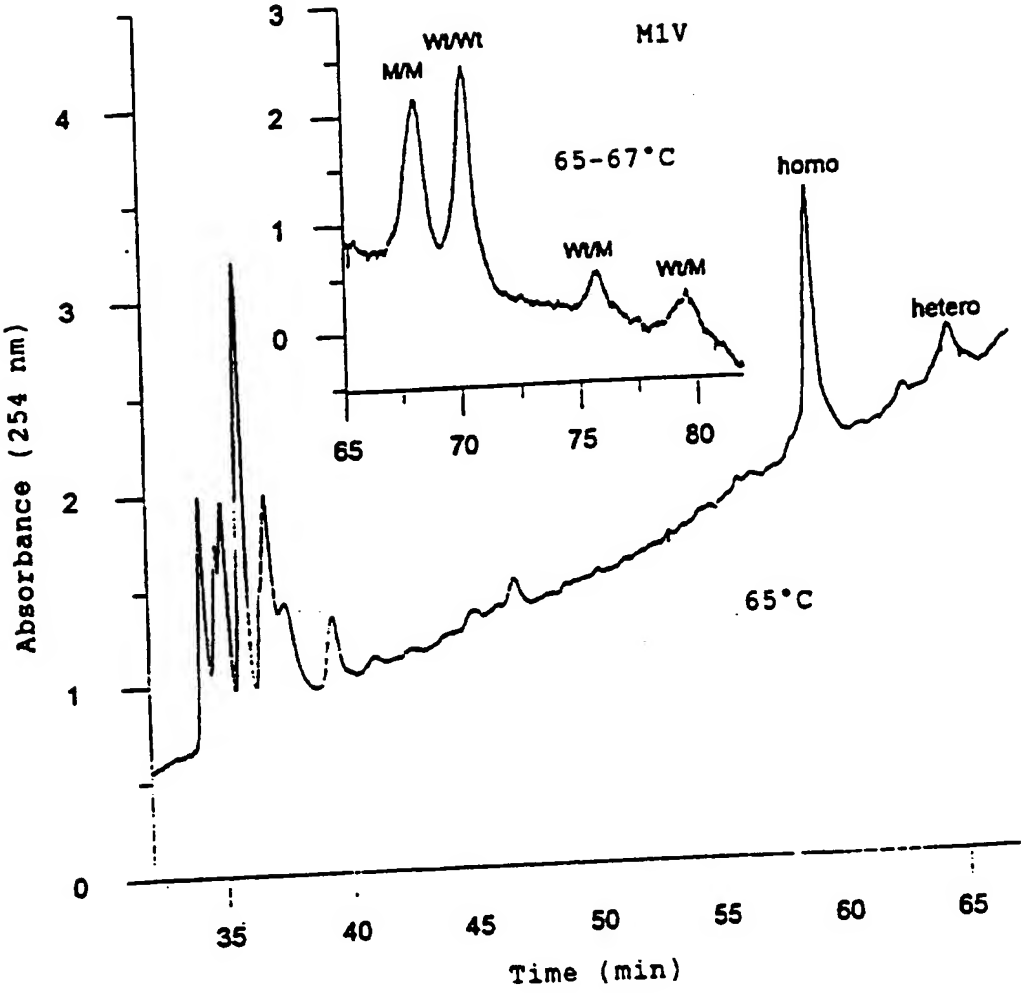
M1V
M/M
W/W
65-67°C
W/M
W/M
homo
hetero
65°C
Absorbance (254 nm)
Time (min)
0
1
2
3
4
35
40
45
50
55
60
65
65
70
75
80

Figure 4

$M_w 10^3$ (---)
1500
1000
500
90
70
50
30
Time (hours)
5000
4000
3000
2000
1000
0
Viscosity (mPa · s) (——)

Figure 5

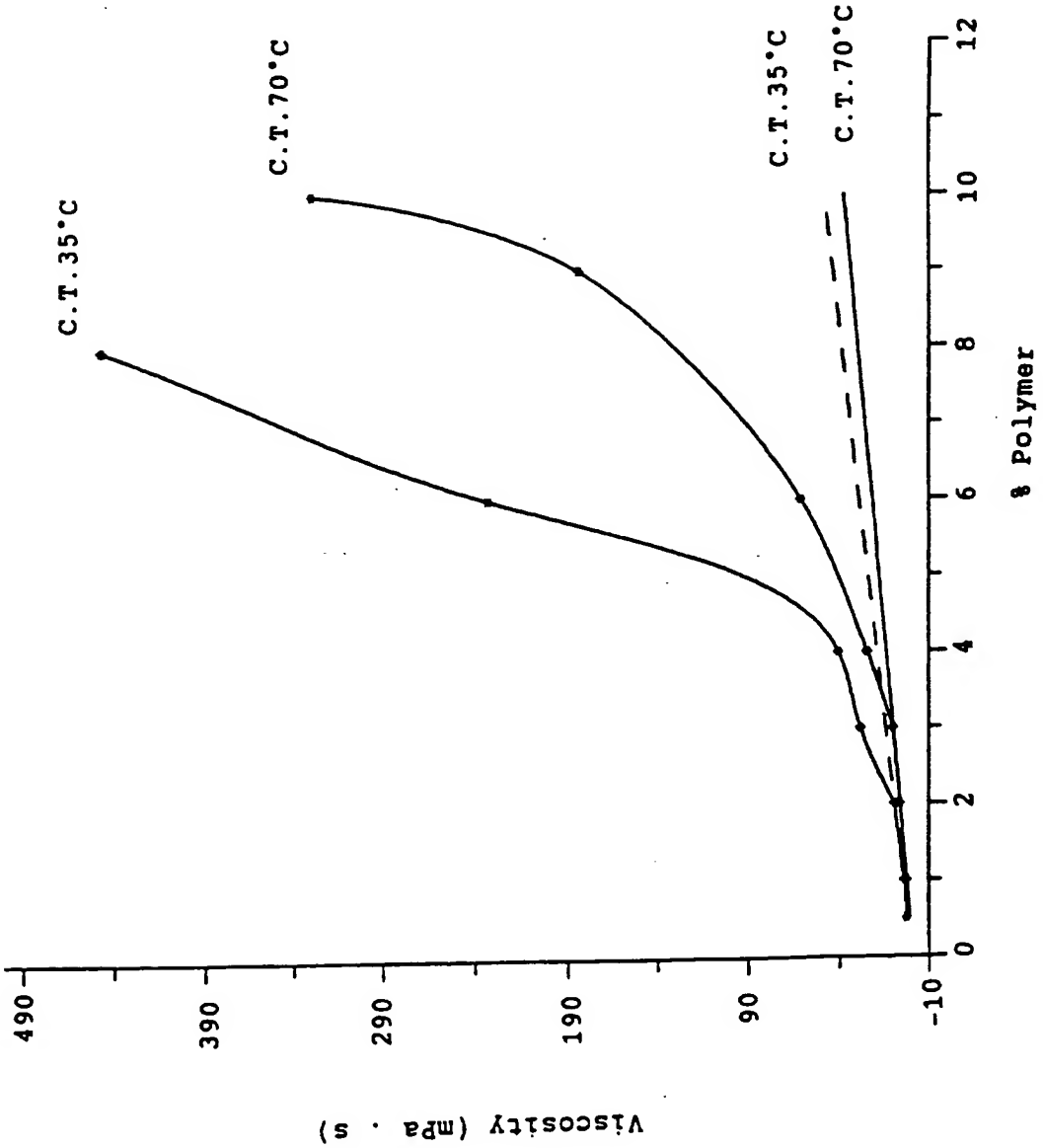
C.T.35°C
C.T.70°C
C.T.35°C
C.T.70°C
490
390
290
190
90
-10
0
2
4
6
8
10
12
% Polymer
Viscosity (mPa · s)

Figure 6

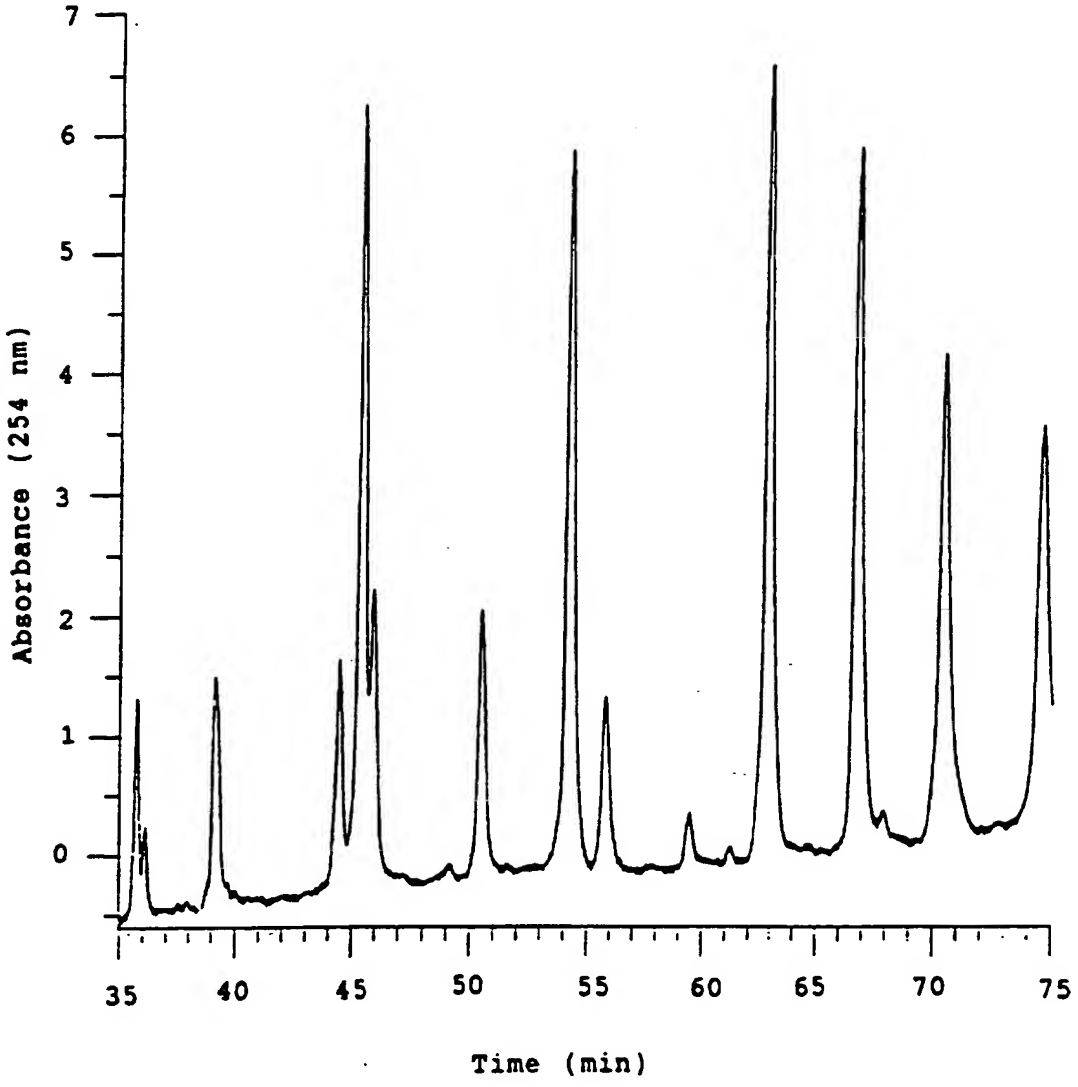

Figure 7A

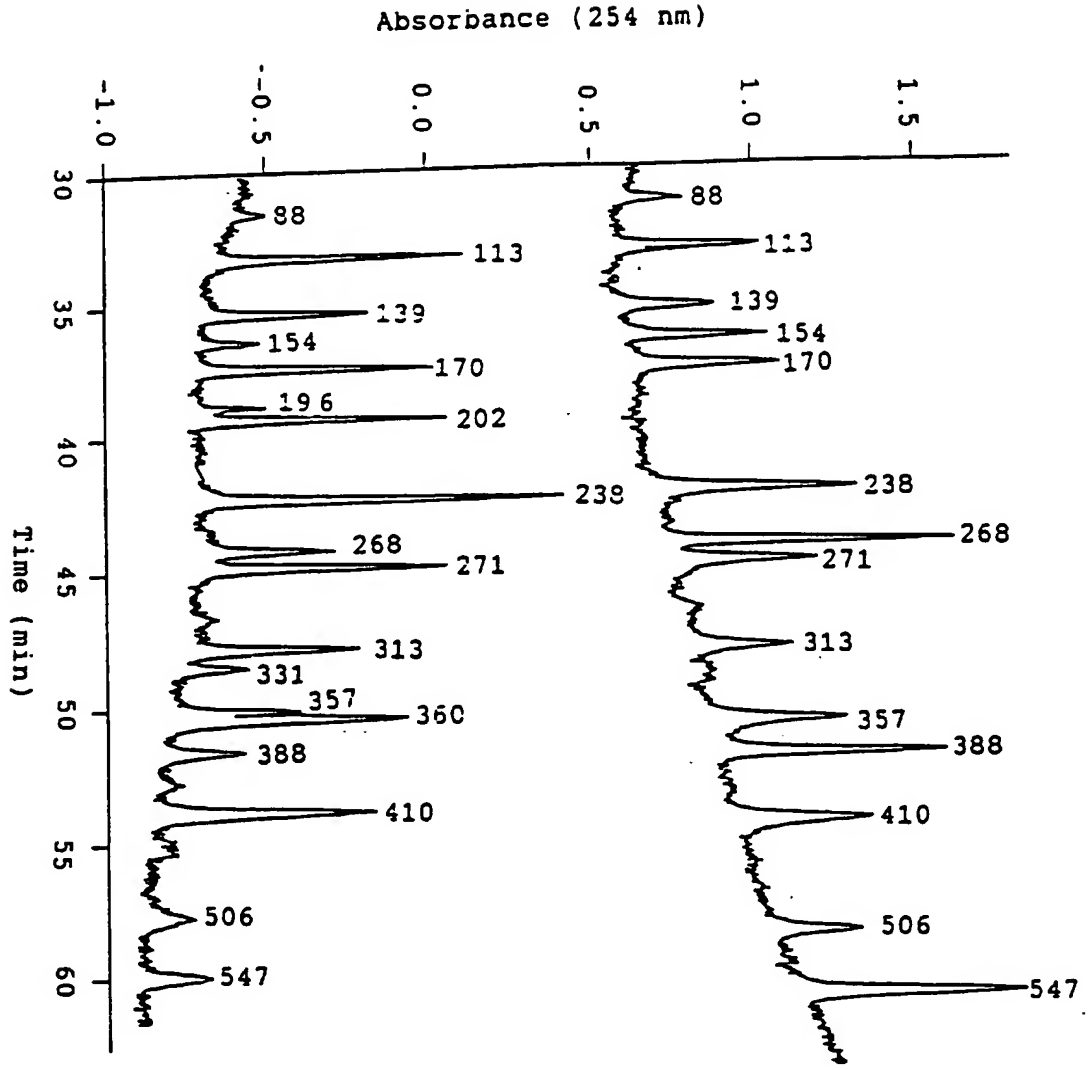
Absorbance (254 nm)
-1.0
-0.5
0.0
0.5
1.0
1.5
Time (min)
30
35
40
45
50
55
60
88
113
139
154
170
196
202
238
268
271
313
331
357
360
388
410
506
547

Figure 7B

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 95/03561

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,91 02815 (DIAGEN INSTITUT FÜR MOLEKULARBIOLOGISCHE DIAGNOSTIK GMBH) 7 March 1991 see page 10, line 13 - page 11, line 23	1
Y	ANALYTICAL CHEMISTRY, vol. 64, no. 8, 15 April 1992 WASHINGTON, DC, US, pages 967-972, XP 000271819 X. C. HUANG 'CAPILLARY ARRAY ELECTROPHORESIS USING LASER-EXCITED CONFOCAL FLUORESCENCE DETECTION' see figure 1	1
A	EP,A,0 329 341 (APPLIED BIOSYSTEMS, INC.) 23 August 1989 see page 5, line 53 - line 58	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
3 January 1996	24.01.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Duchatellier, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/EP 95/03561

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		DE-A-	4006974	12-09-91
		EP-A-	0486580	27-05-92
		JP-T-	5503351	03-06-93
EP-A-329341	23-08-89	AT-T-	121842	15-05-95
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		JP-A-	2010150	12-01-90
		US-A-	5240576	31-08-93
		US-A-	5207886	04-05-93